Pathogenesis and Diseases of the Central Nervous System Caused by Murine Coronaviruses

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I. INTRODUCTION

This chapter is an account of studies of central nervous system (CNS) diseases connected with neurotropic variants of MCV such as J. Howard Muller Virus (JHM) and A59 and deals with animal models that may have relevance to an understanding of human diseases of putative viral etiology such as multiple sclerosis (MS). From the time of (JHNV) isolation from paralyzed mice by Cheever *et al.* (1949) and Bailey *et al.* (1949), this agent and related strains have provided copious data about encephalitic and demyelinating diseases in rodents. To date, however, any possible connection between murine coronavirus (MCV) and MS is tenuous. The reported isolation of coronavirus (CV) particles from MS patients' brain (Burks *et al.*, 1980) or electron microscopic visualization of CV-like particles in brain tissue of one MS patient (Tanaka *et al.*, 1976), require confirmation. An older report of JHNV-induced panencephalitis in monkeys (Kersting and Pette, 1956), however, has been confirmed by Murray *et al.* (1992a) in their description of demyelinating disease in several monkey

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species. This finding, coupled with the identification of viral RNA and protein within demyelinating plaques in human brain tissue from MS patients (Murray *et al.*, 1992b; Stewart *et al.*, 1992), are highly provocative data concerning an involvement of CV in the etiology of MS. However, a cautious evaluation of these results may be in order, in view of the recent demonstration of genomic and antigenic relatedness between the N protein of JHMV and the microtubule-associated protein tau (Pasick *et al.*, 1994).

The disease process associated with CV infection is highly variable, both in animals and at the cellular level, depending on the virus, cell types, and host species. In view of the voluminous literature on the subject, this relatively brief review is concerned primarily with disease processes in rodents infected by murine CVs and emphasizes infections of neural cells and pathogenesis in the CNS.

II. VIRUS-CELL INTERACTIONS RELATED TO PATHOGENESIS AND DISEASE

To comprehend the overall disease process elicited by neurotropic MCV, one should consider three major parameters. First, it is necessary to consider the complex organization of CNS tissue, involving a variety of cell types and lineages, that provides a great diversity of cell-cell interactions. Second, one must consider the highly error-prone synthesis of CV genomes that can undergo a high frequency of recombination, generating rapidly evolving mutants or variants (Lee *et al.*, 1991; Lai, 1990). Such variants manifest altered tropism and virulence, as detailed in Section IV. Third, one must keep in mind the effects of host immunity evoked by CV infections. These responses, primarily of the cellular type, but also to some extent humoral, can modulate pathogenesis and disease, as described in Section III.

A. Correlations between Infections *in Vivo* and with Explanted Neurons and Glia

Infections of different tissues and organs *in vivo* develop according to the MCV type and the route of inoculation employed. The natural means for initiating infection is through intranasal (IN) instillation of virus, but introduction through intraperitoneal (IP) and intracranial (IC) injections is also commonly employed. Neonates and juveniles usually succumb more rapidly than adults, developing fulminant infections manifested by widespread destruction of tissues. In the CNS an early onset acute encephalitis develops.

In adult mice, responses are variable depending on the MCV type and the genetic constitution of the host, as discussed in more detail in Section IV. When adult mice are challenged intranasally with the highly virulent murine hepatitis virus S (MHV-S), occurrence of a rapidly fatal infection of the CNS is associated with generalized virus dissemination to the bowel, liver, spleen, and other organs (Taguchi *et al.*, 1979; Barthold *et al.*, 1986; Lavi *et al.*, 1984a).

Histopathology in the CNS includes spongiform lesions within the brain stem. Comparable IN inoculation of neonates with neurotropic A59 and JHMV also results in a wide dissemination into the CNS, respiratory, and vascular endothelium and elsewhere (Barthold and Smith, 1984).

By contrast, A59 and JHM, when given IN to adult mice, induce milder forms of disease. The viruses progress slowly through the olfactory tracts before spreading into the brain and spinal cord (SC) (Barthold *et al.*, 1986). Encephalitis or subacute paralytic symptoms frequently ensue (Lavi *et al.*, 1988; Barthold *et al.*, 1986). The viscerotropic MHV3 strain of MCV, when inoculated into resistant A/JX mice, fails to produce disease symptoms, but nevertheless replicates in liver and brain (Tardieu *et al.*, 1986). Evidently MCVs belonging to the neurotropic or viscerotropic categories possess a more general tropism in the mouse than is indicated by their designation.

After IN infection of mice, virus dissemination and progress of disease have been followed within the CNS and other tissues by means of antibodies and nucleic acid probes. The localization of viral antigen and RNA by means of *in situ* hybridization in sections has been highly informative. With C57BL/6 mice, in which acute encephalitis develops upon infection with A59 or JHMV, the virus becomes widespread within the cortical gray matter (GM), brain stem, the white matter (WM) tracts in the regions of the optic chiasma, and the SC (Lavi *et al.*, 1988; Perlman *et al.*, 1988, 1989, 1990). A time-course reconstruction of events leads to the conclusion that virus spreads from the trigeminal olfactory locus along anatomically and functionally interconnected neuronal tracts. Dissemination of virus along such neuronal pathways provides a rationale for the speed at which virus can spread toward and within the SC. Virus expression is detectable at the cellular level in neurons and glia, consistent with the histopathology associated with the acute encephalitic or progressive demyelinating diseases caused by A59 and JHM (Perlman *et al.*, 1988; Fleming *et al.*, 1987; Spaan *et al.*, 1988). In the mouse CNS the neurons and oligodendrocytes (OL) are the prominent cell types containing viral RNA and antigen (Lavi *et al.*, 1987), although astrocytes (AS) may also become targets in primary explants. In such cell cultures from neonatal BALB/c or CD.1 mice, both OL and AS are infectable by MCV A59, JHM, and human coronavirus (HCV) OC43 (Wilson *et al.*, 1986; van Berlo *et al.*, 1989; Pearson and Mims, 1985). Likewise, explanted neurons can be productive host cells for A59 and JHMV (Dubois-Dalq *et al.*, 1982; Knobler *et al.*, 1981).

Although the CNS disease process induced in the rat by MCV has many parallels with that observed in mice, notable differences are evident. They include an age-related refractoriness to infection, apparent by the time of weaning (Sorensen *et al.*, 1987a); a requirement for larger inocula, which are usually administered IC to promote efficiency, although neonates can be challenged successfully IN (Hirano *et al.*, 1980); and an association of the CNS and paralytic forms of disease with JHMV but not MHV3, although the latter can produce inapparent infections (Hirano *et al.*, 1980). Again, the progress of infection can be followed in the CNS by antigen and RNA probes, which demonstrate that dissemination occurs in a temporal sequence, culminating with virus spread within the SC (Sorensen *et al.*, 1980, 1984b). The cell types promi-

nently involved in infection include neurons and OL. *In situ* hybridization by means of cDNA probes has pinpointed the presence of viral RNA in hippocampal and cerebellar neurons. The virus may remain sequestered in these targets for prolonged periods, even during episodes of subacute, paralytic disease evident in the Wistar Furth (WF) rat model (Sorensen and Dales, 1985; Parham *et al.*, 1986). Rat CNS explants, cultured to promote neuronal survival *in vitro*, are likewise infectable with JHMV. In such cultures, replication occurs preferentially or exclusively in neurons (Pasick and Dales, 1991). The susceptibility of primary neuron explants to JHMV is consistent with them being primary targets for the development of acute encephalomyelitis (Knobler *et al.*, 1981; Dubois-Dalq *et al.*, 1982; Buchmeier *et al.*, 1984; Sorensen and Dales, 1985; Pasick and Dales, 1991; Matsubara *et al.*, 1991; Zimprich *et al.*, 1991).

Contrary to the dual tropism of JHMV observed with murine cells, in our studies with explanted rat cells, this virus exhibits a reciprocally exclusive tropism for OL compared with MHV3, which infects only type 1 AS (Beushausen and Dales, 1985). This tropism pertains to explants from WF or Wistar Lewis (WL) strains. Our findings were confirmed with Wistar rat glial cultures (van Berlo *et al.*, 1986), although JHMV infection of both OL and AS was detected with glial cultures from WL rat CNS (van Berlo *et al.*, 1989). In this case, the AS lineage (type 1 or 2) was not established.

The inverse tropism to those that we determined has been described in CNS explants from WL rats (Massa *et al.*, 1986). In this study, immunofluorescence labeling indicated selective infection of AS rather than OL, with some modulation in tropism (Massa *et al.*, 1988). These diverse findings on the tropism of JHMV for rat glia have not been reconciled completely, but could be related to the identity of the lineage of infectable AS. The presence of glial acidic fibrillary protein (GFAP) as filament bundles that characterize type 1 AS, also occur, albeit transiently, in the progenitor cell type O2A, from which both OL and type 2 AS differentiate (Raff, 1989; Lillien and Raff, 1990). Since immature, rather than fully differentiated, rat OL appear to be susceptible to JHMV (Beushausen and Dales, 1985), it is likely, as shown by Pasick and Dales (1991), that GFAP⁺ O2A cells become infected and express virus antigen, accounting for the observations of Massa *et al.* (1986, 1988).

In general, the tropism defined *in vitro* matches the observed distribution of CV infections of the CNS *in vivo*, and is also consistent with the pathogenesis and disease processes that become evident.

B. Ligand-Receptor Interactions

The surface component of CV that attaches to receptors on the host cell is the surface glycoprotein. This molecule possesses features generally associated with peplomers of enveloped viruses (Schmidt *et al.*, 1987). Activation of the fusogenic and penetration activities of S is enhanced by proteolytic cleavage into the subunits S₁ and S₂ (Schmidt *et al.*, 1987; Luytjes *et al.*, 1987; Frana *et al.*, 1985; Sturman *et al.*, 1985; Stauber *et al.*, 1993; Taguchi, 1993). However, attachment and neutralization by antibodies are not contingent on the proteolytic

processing of S (Luytjes *et al.*, 1989; Collins *et al.*, 1983). The cell-to-cell spread of infection in the human line Medical Research Council (MRC-C) challenged with HCV 229E (Appleyard and Tisdale, 1985) or the murine cell line 17 Cl I challenged with A59 (Sturman *et al.*, 1985; Frana *et al.*, 1985), requires the activation of S by a cellular protease that is inhibited by agents such as leupeptin (Appleyard and Tisdale, 1985). Also, virus dissemination is not absolutely contingent on the formation of syncytia, even after S has been processed into S₁/S₂. Thus, MHV A59 is highly fusogenic for 17Cl I and L cells, but in contrast to MHV3 and JHMV is nonfusogenic *in vivo* or for explanted rodent glial cells (Lucas *et al.*, 1977; Sturman *et al.*, 1985; Frana *et al.*, 1985; Lavi *et al.*, 1987; Wilson and Dales, 1988). Differences in syncytiogenic activity among CVs remain unexplained.

The receptor specificity for CV has also been studied as a determinant of tropism. In one system using rat glial cultures, one can demonstrate an unambiguous tropism of JHMV for OL and MHV3 for AS, but there is no difference in the binding efficiency of labeled inoculum particles of either virus to either cell type (Beushausen and Dales, 1985). With continuous cell lines such as the rat RN2-2 Schwannoma, which is infectable by JHMV but not by MHV3, both virus types likewise become attached equally well (Lucas *et al.*, 1977, 1978). In the case of the C6 rat astrocytoma cell line, which is resistant to infection by both viruses, receptors for JHMV, MHV3, and other MCV strains are present (van Dinter and Flintoff, 1987; Flintoff and van Dinter, 1989; Kooi *et al.*, 1988). In the case of sublines derived from the prototype L strain murine line, the more restrictive subline LM-K can absorb A59 virus as efficiently as the highly permissive L2 cell (Kooi *et al.*, 1988). A similar situation is found with cells from SJL/J mice, which are highly resistant to JHMV but not to MHV3. Thus, both viruses can be adsorbed with the same efficiency to OL and AS of SJL/J mice and to OL and AS of permissive BALB/c and CD.1 mice (Wilson *et al.*, 1986).

A presumed absence of receptors for A59 on SJL/J hepatocytes and enterocytes, which was assumed to account for differences in resistance of SJL/J mice compared to susceptible BALB/c mice (Boyle *et al.*, 1987), also can now be explained. The MHV receptor is a 110-120-kDa G-P, a member of the carcino-embryonic antigen family (Williams *et al.*, 1991). Although this G-P is expressed on SJL/J cells, its form is modified (Yokomori and Lai, 1992), presumably affecting the efficiency of JHMV replication. Whatever the deficiency of receptors for JHMV and A59 might be in SJL/J mice, the resistance of neurons and glia from these mice is due to a defect in cell-cell virus spread rather than an absence of receptors per se (Wilson and Dales, 1988; Pasick *et al.*, 1992). Virus dissemination may involve a deficiency in the processing of S because a more fusogenic JHMV variant ATf11, isolated from rat SC (Morris *et al.*, 1989), is not restricted in SJL/J glial cells (Pasick *et al.*, 1992).

The identification of a hemagglutinin esterase (HE) glycoprotein component in BCV (Vlasak *et al.*, 1988) drew attention to the possible function of HE during early interactions with host cells. By sequence comparison, HE shows evolutionary relatedness with the influenza type C hemagglutinin H₁ subunit (Kienzle *et al.*, 1990). The esterase of HE has receptor-binding activity and

inactivates receptors on erythrocytes by hydrolyzing O-linked acetylsialic acid (Schultze *et al.*, 1991). Since this reaction and virus entry are both blocked with diisopropyl fluorophosphate (Vlasak *et al.*, 1988), it is presumed that HE acts in a manner similar to that of a receptor-destroying enzyme of type C influenza virus (Kienzle *et al.*, 1990). Evidently the esterase is not essential for infectivity because it is absent from MCV A59 and some MCV JHM strains (Parker *et al.*, 1990). Some of the roles suggested for HE are: as a determinant of tropism or virulence in the CNS; as an additional ligand for binding of cell targets; or for facilitating the penetration of inoculum virus (reviewed in Parker *et al.*, 1990; Vlasak *et al.*, 1988).

C. Penetration

An electron microscopic examination of the interaction between surfaces covering the microvilli in calf intestine inoculated with the calf diarrhea CV was interpreted as showing fusion between the virus envelope and plasma membrane on microvilli (Doughri *et al.*, 1976). Studies on cells in culture, using biochemical and electron microscopic methods, suggested that MCV initiates an infection following sequestration or viropexis of the inoculum (reviewed in Krzystyniak and Dupuy, 1984). The inhibition of early stages of A59 or MHV3 infection, including the eclipse phase (Mizzen *et al.*, 1985), in L2 cells treated with agents such as NH_4Cl and chloroquine (Krzystyniak and Dupuy, 1984), also supports a requirement for engulfment as an obligatory function for initiating infection. More recent unpublished data from our laboratories indicate that NH_4Cl only marginally inhibits the early stages of MHV infection, implying that penetration and release of the genome occurs in "early" endosomes containing a neutral or only slightly acidic milieu due to their intracellular location close to the cell surface. (Schmid *et al.*, 1989).

Studies of penetration by JHM, A59, and MHV3 into continuous neural and nonneural cell lines suggest that interactions with the host may vary. The virions that are absorbed onto and sequestered by C6 astrocytoma cells do not become eclipsed (van Dinter and Flintoff, 1987), due to an unidentified defect that can be circumvented by inducing fusion between viral and cell membranes by means of polyethylene glycol (PEG). PEG by itself does not enhance the infectiousness of permissive cells, among them L, RN2-2 Schwannoma, and HTC hepatoma lines. The failure of a "low pH shock" to promote infectability of MCV in the manner observed with vesicular stomatitis virus (VSV) and A-type influenza viruses also argues against involvement of an acidic endosomal compartment in the penetration sequence (Flintoff and van Dinter, 1989). Host control over virus penetration, examined by means of cell-cell hybrids between the resistant C6 and either partially or fully permissive cell lines showed that $\text{C6} \times \text{L2}$ and $\text{RN-2} \times \text{L2}$ cell lines could be infected without the use of PEG (Flintoff and van Dinter, 1989; Coulter-Mackie *et al.*, 1984). However, these permissive cell hybrids retain the phenotype of the parent that resists extensive syncytia formation. This demonstrates that penetration and MCV-induced cell-cell fusion are controlled independently.

D. Uncoating and Initiation of Genome Functions

Although the ssRNA genome penetrates from inoculum virions into the host within a helical coat of N protein (Davies *et al.*, 1981), the infectivity of the naked genomic RNA (Siddell *et al.*, 1982) implies that, in the normal course of penetration, the uncoating step involves dissociation of N from the RNA. The nascent N polypeptide of JHMV has a molecular weight of 50 kDa which increases to 56 kDa upon phosphorylation. The 56-kDa phosphorylated form of N exists in virions (Armstrong *et al.*, 1983; Wilbur *et al.*, 1986; Siddell *et al.*, 1981). From sequence data on N (Armstrong *et al.*, 1983; Skinner and Siddell, 1983) and other evidence (Wilbur *et al.*, 1986), one can deduce that N has two to four, or perhaps more, potential sites for phosphorylation, a modification effected prior to virion assembly by either cellular kinases (PK) or a MCV-specified PK. In a converse sequence of events taking place during penetration, the molecular weight of N is reduced initially from 56 to 50 kDa, then further breakdown into smaller molecular weight polypeptide fragments occurs (Beushausen *et al.*, 1987). Conceivably, dephosphorylation of N promotes the uncoating process, as documented for retroviruses (Beushausen *et al.*, 1987; Mohandas and Dales, 1991).

Our demonstration of the existence, in an endosome fraction of a cellular phosphoprotein phosphatase [(PPPase) belonging to the neutral type 1 class of enzyme, unpublished], with a high specificity for N (Mohandas and Dales, 1990), is consistent with the view that uncoating may either commence or proceed to completion in "early" endosomes and/or close to the cell surface. In this context, it is worth mentioning that endosomal PPPases of a type similar to that acting on N have been recognized in clathrin-coated vesicles, where they may catalyze the rupturing of polymeric clathrin protein cages and facilitate movement of the freed endosomes and fusion of the endosomal membrane with other membrane compartments (Pauloin *et al.*, 1988; Greene and Eisenberg, 1988; Loeb *et al.*, 1989).

E. Effect on Cellular Protein Synthesis

Viruses have developed diverse strategies to ensure the translation of virus-specific mRNAs in the infected cell. MCV gives rise to mRNAs that share the structural characteristics of most eukaryotic mRNAs, such as the presence of 5' terminal caps (Lai *et al.*, 1982) and 3' poly A tails (Cheley *et al.*, 1981; Cheley and Anderson, 1981). It appears unlikely, therefore, that translational control in MCV infection is mediated by the alteration of host initiation factors such as occurs, for example, in poliovirus infection and results in the selective translation of structurally distinct (e.g., uncapped) mRNAs (Kaufman *et al.*, 1976; Trachsel *et al.*, 1980).

At high multiplicities of infection, MCV inhibits host protein synthesis in certain cell lines. In mouse L2 fibroblasts, infection results in a decline in total protein synthesis to about 7% of controls. This overall decrease is likely due to a partial block of translation at the initiation stage, as indicated by an

increase in free 80S ribosomes. There is also a shift in size from heavier to lighter polysomes, reflecting increased competition by a greater number of shorter mRNAs for a limited quantity of available ribosomes. The overall total poly(A)⁺RNA content of the infected cell is increased approximately threefold by 6 hr postinfection, as a result of the synthesis and accumulation of viral mRNA. Most interestingly, by this time, and later in infection, cellular mRNA transcripts disappear, apparently due to a degradative mechanism (Hilton *et al.*, 1986).

Previously, virus-induced hydrolysis of host mRNA had been noted after infection with complex DNA viruses, such as herpes simplex (Nishioka and Silverstein, 1978) and vaccinia (Rice and Roberts, 1983), and with influenza, an RNA virus. In the case of influenza, breakdown of host mRNA is a consequence of the unique mode of transcribing, i.e., the capture of host-derived 5'-caps (Bouloy *et al.*, 1980; Plotch *et al.*, 1981). Most other RNA viruses studied to date, however, appear to effect translational controls by nondegradative mechanisms (Fernandez-Munoz and Darnell, 1976; Gallwitz *et al.*, 1977; Lodish and Porter, 1981). Evidence has also been presented suggesting that during infections with some viruses, including adenovirus (Lazaridis *et al.*, 1988) and La Crosse virus (Raju and Kolakofsky, 1988), viral functions are expressed that affect the stability of cellular or viral mRNAs.

It is intriguing to speculate that the mechanism of RNA degradation connected with MCV infection (Hilton *et al.*, 1986) is related to the unique replication strategy of coronavirus. It has been suggested that the synthesis of CV subgenomic mRNAs may involve nucleolytic trimming (Lai, 1986). Although this type of nucleolytic activity has yet to be identified, if present, such a MCV-induced nuclease could also presumably recognize as a substrate cleavage sites in cellular mRNAs, thereby accelerating their hydrolysis by host degradative mechanisms.

Another obvious cell-killing function of MCV infection is the inhibition of host translation (e.g., Marvaldi *et al.*, 1977). Differences among host cells in their susceptibility to MCV-mediated translational control could be manifested in the cytopathology that occurs and also determine whether the outcome is an acute or persistent infection.

F. Effects on Cellular Membranes

The unique sites of CV assembly by budding from smooth endoplasmic reticulum membranes, recognized by electron microscopy (Massalski *et al.*, 1982; Barthold and Smith, 1984; Pasick *et al.*, 1994; Krijnse-Locker *et al.*, 1994; Klumperman *et al.*, 1994), has not yet been explained. Earlier, it was thought that the M protein had an important role in organizing the assembly of virion envelopes on these internal membranes, mainly because this protein has signals for retention in the Golgi (Swift and Machamer, 1991). Recently, however, it has become apparent that the Golgi compartment is beyond the site of budding (Klumperman *et al.*, 1994). One reason suggested for M being anchored and retained on internal membranes is the preferential O-linked glycosylation

(Rottier *et al.*, 1984; Niemann and Klenk, 1981; Niemann *et al.*, 1984). In contrast, the S protein, which possesses N-linked carbohydrates, behaves like the usual peplomer G-P of viruses budding from the cell surface. Thus, although S attaches to the formative CV envelopes within the budding compartment, it can also migrate independently to the plasma membrane where it accumulates and can express fusogenic activity (Rottier *et al.*, 1984; Niemann *et al.*, 1984).

Electron microscopic examination of MCV-infected cells has revealed apparent distensions or amplification of intracellular membranes that may be derived from smooth endoplasmic reticulum (David-Ferreira and Manaker, 1965; Massalski *et al.*, 1982; Dubois-Dalq *et al.*, 1982; van Berlo *et al.*, 1986; Tooze *et al.*, 1984). It has been speculated that such membrane proliferation or reorganization may reflect a cellular response to the removal of membrane lipid as it becomes incorporated into virion envelopes (Tooze *et al.*, 1984). This explanation seems to be unlikely since membrane proliferation has been observed during infection with a temperature-sensitive (*ts*) mutant of MCV, when virion assembly was interrupted (van Berlo *et al.*, 1986). The stimulation of membrane proliferation and lipid biosynthesis resulting from cytopathology have also been observed following infections with other agents, among them nonenveloped polio- and mengovirus (Penman, 1965; Amako and Dales, 1967).

A very obvious manifestation of virus-cell membrane interactions occurring within the replication cycle of MCV is membrane fusion. Fusion occurs both early during virion entry and uncoating and later when syncytiogenesis is evident. The control of cell-cell fusion is closely related to events that contribute to the establishment of a cytolytic versus an attenuated or persistent infection (Kooi *et al.*, 1988; Mizzen *et al.*, 1983, 1985, 1987b).

The S protein is a key mediator for both cell fusion and pathogenesis. Within the infected CNS, differences among cell types have been noted in relation to virus-induced cell fusion (Sorensen *et al.*, 1980; Dubois-Dalcq *et al.*, 1982; Buchmeier *et al.*, 1984). By inducing cell-cell fusion from within, MCV may facilitate both dissemination of virus and maintenance of persistent infections, as demonstrated in the presence of neutralizing antibodies added to the medium (Buchmeier *et al.*, 1984; Sorensen *et al.*, 1984a; Stohlman and Weiner, 1978). In cultures, such as the Cl-1 line, which is relatively resistant to JHMV-mediated syncytiogenesis, individual cells may survive a cytolytic, acute infection, thereby maintaining a state of virus persistence (Mizzen *et al.*, 1983).

The resistance of murine cells to various strains of MCV appears to be under the control of a single autosomal, recessive gene (Bang and Warwick, 1960; Virelizier and Allison, 1976; Stohlman and Frelinger, 1978). Concomitantly, the complete or partial genetic resistance to disease observed with some strains of mice is inherent in explanted macrophages, hepatocytes, fibroblasts, and other cell types. The infection of cells from such mice results in either an abortive (Virelizier and Allison, 1976) or limited infection, with reduced cytopathology (MacNaughton and Patterson, 1980; Arnheiter *et al.*, 1982; Lamontagne and Dupuy, 1984a). Although cultured macrophages, OL and AS from SJL/J mice, which are resistant to JHMV as primary targets of infection, can support MCV replication, the cell-cell spread of virus due to a fusion-dependent mechanism is restricted (Knobler *et al.*, 1984). Some deficiency of an SJL/J cell protease

was suggested as an explanation for the absence of cell-cell spread of virus. In fact, studies on the spontaneous acquisition of resistance by L2 cell mutants, selected for the survival of a usually lethal MCV infection, demonstrate that partial resistance to infection and to S-mediated syncytia formation are comutable characteristics of the host cell (Daya *et al.*, 1989). Homeostatic control of fatty acid and cholesterol metabolism has been shown to be another important regulator of virus-mediated cell fusion both *in vivo* and *in vitro* (Cervin and Anderson, 1991).

It has been assumed that differences in the infectability and susceptibility of cells to MCV-mediated fusion are correlated (Mizzen *et al.*, 1983, 1987b), and depend on proteolytic activation of S, presumably involving a host cell protease (Frana *et al.*, 1985). Quantitative cleavage of S is, however, not necessary for cell-cell fusion, and there is no strict correlation between the proteolysis of S and the extent of cell fusion (Frana *et al.*, 1985), as indicated when S was mutated in the cleavage signal and expressed independently by vaccinia recombinants (Stauber *et al.*, 1993; Taguchi, 1993). Also, one class of fusion (from within) defective MCV mutant, which possesses a single mutation within the cleavage signal of S, has been isolated from persistently infected glial cultures (Gombold *et al.*, 1993). It is possible that uncleaved S protein is fusogenic, but that fusogenicity is enhanced upon proteolytic cleavage.

CV-induced membrane fusion and the severity of infection are also influenced by host cell membrane lipid composition (Daya *et al.*, 1988), particularly the cholesterol content (Daya *et al.*, 1988). Conceivably there may be a specific interaction between peptide sequences of the S protein and cholesterol, in a manner similar to that observed with Sendai virus F protein (Asano and Asano, 1988). Another modulation of MCV infection by cholesterol may pertain to the inhibition of Kupffer cell function during *in vivo* infection (Pereira *et al.*, 1987). The observed association of the MCV N protein with cellular membranes may be another example of lipid-dependent events during replication. N is synthesized on free polysomes but associates posttranslationally with membranes, possibly as a result of its phosphorylation (Stohlman *et al.*, 1983). Since N binds the viral M protein (Sturman *et al.*, 1980) and the M protein is found in association with smooth endoplasmic reticulum (ER) membranes (Rottier and Rose, 1987), the membrane attachment may be mediated by the M. Nevertheless, N protein is able to associate with cellular membranes in the absence of M (Anderson and Wong, 1993), raising the possibility that the components involved are certain membrane phospholipids (Anderson and Wong, 1993). Interestingly, the ability of N to bind both membrane phospholipids and nucleic acid is perhaps analogous to that shown by certain bacterial DNA-binding proteins such as DnaA (Sekimizu and Kornberg, 1988) and RecA (Krishna and van de Sande, 1990). The possibility, therefore, arises that an association of N with viral RNA is influenced by competitive binding to membrane phospholipids.

The involvement of the cytoskeleton in a number of MCV infections has also been investigated. Treatment of L2 fibroblasts with colchicine or cytochalasin B, drugs that disorganize microtubules and actin filaments, alters cell shape without affecting syncytiogenesis or the acute cytopathology (Daya *et al.*, 1988). By contrast, in peritoneal macrophages exposed to cytochalasin B, the

infection is changed from an acute to a persistent type (Mallucci and Edwards, 1982). Quite possibly, the cytoskeleton plays a role in regulating MCV assembly and cytopathology, involving membranes that may vary in a cell-dependent manner according to differences in the organization of the cytoskeleton. The close association of N of JHMV with microtubules in neurites of cultured neurons, recently recognized by Pasick *et al.*, (1994), was explained in terms of a specific binding by N, via a region of the protein that has a close sequence homology to the microtubule-binding site of the cytoskeletal protein tau.

As mentioned above, the presence of S at the cell surface is related to cell fusion (Collins *et al.*, 1982). However, it may also affect control of plasma membrane permeability, as evidenced by an influx of sodium ions (Mizzen *et al.*, 1987a). This occurs during both acute and persistent infections (MacIntyre *et al.*, 1989). However, in contrast to certain other virus infections (Carrasco and Smith, 1976), there is evidence that in MCV-infected cells an elevated intracellular sodium ion concentration affects the regulation of translational control (Mizzen *et al.*, 1987a). One should not exclude the possibility that altered membrane permeability can affect other aspects of viral cytopathology.

G. Factors Related to Latency and Persistence

Infection of the CNS can establish a very prolonged state of CV latency or persistence. Whether the animal remains asymptomatic or undergoes a late-onset progressive paralytic disease with demyelination, virus expression in the form of RNA, antigen, or infectious particles may continue for prolonged periods in mice (Pickel *et al.*, 1981; Lavi *et al.*, 1984a,b, 1987; Perlman *et al.*, 1988; Knobler *et al.*, 1981) or rats (Wege *et al.*, 1983, 1984; Sorensen *et al.*, 1984b; Sorensen and Dales, 1985). Persistent infections are readily established in explanted primary neural cells (Dubois-Dalq *et al.*, 1982; Beushausen and Dales, 1985; Wilson *et al.*, 1986; Pearson and Mims, 1985; van Berlo *et al.*, 1989; Lavi *et al.*, 1987) and continuous cell lines of neural and nonneural derivation (Flintoff and van Dinter, 1989; van Dinter and Flintoff, 1987; Coulter-Mackie *et al.*, 1984, 1985; Lucas *et al.*, 1977; 1978; Collins and Sorensen, 1986; Leibowitz *et al.*, 1984). Evidently, conditions prevail in the CNS of postweanling and adult rodents, as well as in neural cells in culture, which favor maintenance of the state of persistence. Analogous observations have been documented for other neurotropic RNA viruses such as measles virus (Miller and Carrigan, 1982; Yoshikawa and Yamanouchi, 1984), canine distemper virus (Pearce-Kelling *et al.*, 1990; Zurbriggen *et al.*, 1987), rubella virus (van Alstyne and Paty, 1983), picorna viruses (Rodriguez *et al.*, 1990), and alpha viruses (Bruce *et al.*, 1984). A common feature linking MCV with all of the above agents is an initial low rate of replication associated with limited cytopathology. Following a period of adaptation, during which, perhaps, variants are selected and maintained, the infectious process may remain unchanged or switch into a more acute or more attenuated mode (Taguchi and Siddell, 1985) or replication may be entirely suppressed (Collins and Sorensen, 1986). The exceptionally high efficiency of MCV genetic recombination creates numerous variants (Lai *et al.*, 1985; Ma-

kino and Lai, 1989), a phenomenon that can account, at least partially, for the varying types of infection encountered, as discussed in Section IV.

The repeated passage of JHMV in murine fibroblastic DBT cell lines generates defective-interfering (DI) particles in which genomes are shortened by deletions (Makino *et al.*, 1984, 1985). In a high multiplicity of infection, DIs interfere with the transcription of all mRNAs species with the exception of mRNA7, coding for N. There is no substantial evidence linking DIs with CV infections of the CNS or neural cell explants (Taguchi and Siddell, 1985).

A regular feature of MCV persistence in continuous cell lines, not exclusively of the neural type, is a wavelike, cyclical production of infectious particles (Lucas *et al.*, 1977, 1978; Coulter-Mackie *et al.*, 1984, 1985). Such periodicity in virus titer also has been reported for measles and rabies viruses (reviewed in Lucas *et al.*, 1978) but is not evident during MCV infections of primary neural cell cultures (Beushausen and Dales, 1985; Wilson *et al.*, 1986). With rabies virus, the rise and fall of titers was explained as due to reciprocal periodic development of an antiviral state related to the autocrine activity of interferon (IFN) (reviewed in Coulter-Mackie *et al.*, 1985). During persistent infection of porcine alveolar macrophages by transmissible gastroenteritis virus (TGEV) of pigs, an induction of α -IFN was observed (Laude *et al.*, 1984). By contrast, our extensive, detailed search for the induction of IFN during a cyclical rise and fall of JHMV titers from RN2-2 Schwannoma cells failed to detect the presence of this lymphokine, although these host cells have the capacity to acquire an antiviral state and make IFN when appropriately induced with reovirus (Coulter-Mackie *et al.*, 1985). Likewise, persistent infections of mouse neuroblastoma C1300 cells with JHMV (Leibowitz *et al.*, 1984) and human glioblastoma U-87 HG cells infected with HCV OC43 (Collins and Sorensen, 1986) do not evoke the production of IFN, demonstrating that in these systems IFN or the antiviral state are not required to maintain persistence. The effectiveness of IFN as a protective factor may also depend on the MCV strain involved. Thus, with DBT cells, which become productively infected, α -IFN fails to arrest replication or the development of cytopathology when the highly virulent MHV-2 is used, whereas IFN treatment can abrogate both virus production and cell killing when MHV-S or JHMV are used as infectious agents (Taguchi and Siddell, 1985).

Another reversible cellular response to MCV infections of primary neural cell explants and continuous cell lines is the suppression of persistent virus production that occurs on a shift up of the ambient temperature from a permissive 32–33 °C to the restrictive 39–40 °C (Lucas *et al.*, 1977, 1978; Coulter-Mackie *et al.*, 1984, 1985; Sorensen *et al.*, 1984a). The MHV3 and JHMV progeny generated during permissive infection of L2 cells do not acquire or possess a thermosensitive or thermolabile phenotype during passage through lymphocytic (Lamontagne and Dupuy, 1984b) or neural cells (Lucas *et al.*, 1978; Leibowitz *et al.*, 1984). Similar results were obtained with measles virus (Lucas *et al.*, 1978). This implies that latency at elevated temperature is due to some controlling factor(s) in the host cells per se that is not IFN (Coulter-Mackie *et al.*, 1985; Leibowitz *et al.*, 1984). By contrast, replication of HCV OC43 in the UH87 HG line generates thermosensitive variants, so that during restriction at

high temperature persistent or latent OC43 virus is rapidly eliminated and cells are "cured" (Collins and Sorensen, 1986).

A detailed study of latency was undertaken using the RN2-2 cells infected with JHMV (Coulter-Mackie *et al.*, 1985). The virus formed during persistent infections at 33 °C is not temperature sensitive. Within 24 hr after shift up to 39 °C, replication is arrested, but the latent genomes are maintained in the host cell for prolonged periods and gene expression, measured as translation into N protein, can be detected for several days. If the latently infected cells, which continue to grow and divide at 39 °C, are returned to the permissive temperature following 2 to 3 weeks, virus production slowly resumes. Analyses made by following a time-course in the decline of infectious centers yielded data compatible with a probability model that predicts that due to continual segregation of the limited number of latent JHMV genomes among daughter cells, the virus genome is eventually lost by a dilution effect.

III. RELATIONSHIP BETWEEN DEVELOPMENT AND DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM AND THE DISEASE PROCESS

A. Characteristics of the Cells Involved

Under normal circumstances, the susceptibility of rats to CV-induced disease extends only to the time of weaning (Sorensen *et al.*, 1980), whereas mice remain susceptible to challenge into adulthood. The idea that the tropism of CV for specific cells in the CNS can explain the basis of encephalitic and demyelinating paralytic cells in the CNS has already been considered in Section II. In the rat model, a temporal coincidence between the completion of myelination and the onset of resistance to JHMV drew our attention to OL maturation as a possible determinant of resistance to JHMV-induced demyelinating disease (Beushausen and Dales, 1985; Wilson *et al.*, 1986; Beushausen *et al.*, 1987). Also, the availability of methods for reproducibly culturing cells explanted from the CNS now makes it possible to follow the process of differentiation and infection among defined types of neural cells.

Soon after explantation from the brain, a mixture of neurons, cells of glial lineage, and other elements are present in culture. The composition of the nutrient media, including a serum supplement, can profoundly affect the cell types that remain during prolonged cultivation and the cell type selection (Fabre *et al.*, 1985; Gard and Pfeiffer, 1989; Muraoka and Takahashi, 1989; Koper *et al.*, 1984). Thus, with respect to glial cultures, type 1 AS, identified by the presence of GFAP, and the O2A bipotential OL and type 2 AS progenitor cells, which have receptors for monoclonal antibody (MAb) A2B5 (and hence are designated as A2B5⁺) (Raff, 1989), are the most numerous elements when neonatal cortex is explanted into nutrient medium with added serum (Beushausen and Dales, 1985; Koper *et al.*, 1984). Moreover, the progression of O2A cells into AS or OL lineages, respectively, can be reconstructed by following the progeny of individual cells in rat optic nerve cultures (Raff, 1989). These elegant analyses

demonstrated that, even after their separation from the CNS, glial cells are endowed with a predetermined developmental "time-clock" that probably simulates the program existing *in vivo*. The acquisition of GFAP by daughters of O2A parents identifies them as type 2 AS. Daughters of O2A expressing myelin basic protein (MBP), CNPase activity, and GalC on their surfaces are destined to become OL.

The appearance of the mature OL phenotype is more rapid if cells are sparsely distributed in culture dishes (Aloisi *et al.*, 1988) or are grown on poly L lysine, which promotes tight adherence to the substrate. Factors promoting differentiation favor the onset of resistance to JHMV infection (Beushausen and Dales, 1985). Conversion from the O2A into 2AS or OL types is also influenced by growth factors such as basic fibroblastic growth factor (bFGF) and platelet-derived growth factor (PDGF) (McKinnon *et al.*, 1990; Böglér *et al.*, 1990). For example, PDGF, secreted by type 1 AS, promotes the appearance of OL (Raff, 1989; Levine, 1989). When recently established cultures are switched from a serum containing medium to a defined, serumless medium, the survival of neurons is promoted and an alteration of A2B5⁺ cells into differentiated OL is enhanced (Raff, 1989; Levine 1989; Koper *et al.*, 1984). This suggests that there are factors in serum that help to maintain O2A cells but may inhibit the survival of neurons. Interactions between neurons and OL are indicated by the myelination of neuronal processes *in vitro*, albeit of a rudimentary type (Muroka and Takahashi, 1989; Pasick and Dales, 1991). Myelin elaboration in cultures of OL also has been reported in the absence of neurons, implying that axons are not an essential stimulus for triggering myelin formation (Fabre *et al.*, 1985).

B. The Infectious Process in Differentiating OL

If kept for several days, cultures of mixed rat glial cell types contain tightly adherent type 1 AS on the bottom and more loosely attached O2A and OL above the subjacent AS layer. The mixed glia culture can be infected persistently with JHMV and MHV3. After shaking off the top cell layer, type 1 AS are infectable exclusively by MHV3. After replanting, the shaken cells can support only JHMV replication (Beushausen and Dales, 1985; Wilson *et al.*, 1986). Shaken cells, when kept in culture for 2 to 3 weeks, become resistant to JHMV and switch from A2B5⁺ into the mature OL phenotype. Shaken cells can be induced to differentiate more rapidly and resist JHMV infection by pretreatment with N₆,O₂-dibutyryl 3':5' cAMP (dbcAMP) (see Table I). By contrast, AS treated with this metabolite continue to produce abundant quantities of MHV3. Cultured neurons exposed in the same manner to dbcAMP can be infected persistently and produce high titers of JHMV (Pasick and Dales, 1991). With other neurotropic viruses quite different effects of dbcAMP are observed. Infection of AS with rubella virus becomes latent, but it is induced to a productive infection by dbcAMP (van Alstyne and Paty, 1983). In contrast, infection of neuronal and other cell lines with measles virus is reversibly inhibited by dbcAMP, possibly at a late stage in virus expression (Miller and Carrigan, 1982; Yoshikawa and

TABLE I. Effect of dbcAMP on the Replication of JHMV in Primary Oligodendrocytes^a

Treatment of culture	Days postinfection						
	2	3	4	6	8	12	14
Control	13	46.8	55	38.8	720	31	20
1 mM postinfection	33.4	100 ^b	15.5	100	500	26	30
1 mM 48 hr before infection	0	0	0	0	0	0	0
1 mM 48 hr before infection	0	0 ^c	0	0	0	0	0

NOTE: All titers expressed $\times 10^2$ PFU/ml multiplicity of infection 0.5–1.0/cell in each case.

^aAdapted from Beushausen and Dales (1985).

^bdbcAMP added.

^cdbcAMP removed.

Yamanouchi, 1984). These observations indicate that dbcAMP effects may be different for each type of neural cell and virus tested, even among MCV strains.

The resistance to JHMV in cells belonging to the OL lineage can be affected by metabolites other than dbcAMP, as long as they can act to elevate intracellular concentrations of cAMP (Beushausen *et al.*, 1987) (Fig. 1). These data indicate that adenylate cyclase metabolism is generally involved, perhaps through the cAMP-dependent protein kinase(s) (PK). Oligodendrocytes, like brain tissue generally, constitutively possess the PK II activity but lack PK I. A striking effect of induced OL differentiation is a tenfold increase in the amount of PK I regulatory subunit RI despite the absence of constitutive PK I enzyme from OL (Beushausen *et al.*, 1987). Infection by MCV is arrested in mature OL at some stage after internalization of the inoculum but prior to the initiation of genome expression. The critical step affected by dbcAMP treatment, or by the normal differentiation process, could be the uncoating step, defined here as the separation of genome RNA from a phosphorylated form of N constituting the helical capsid (Beushausen *et al.*, 1987). Evidence has been obtained showing that dephosphorylation of N is followed by its proteolytic breakdown. This step, which likely occurs after attachment and uptake, as illustrated in Fig. 2, may be catalyzed by a neutral PPPase concentrated in endosomes (Beushausen *et al.*, 1987; Mohandas and Dales, 1991). A PPPase that is highly specific for N occurs in rat brain tissue, OL ROC-1 cells (a hybrid cell line created from OL $\times$ C6), and in the highly permissive L2 cell (Mohandas and Dales, 1990). It is remarkable that the N-specific, endosomal PPPase activity is inhibited by the addition of RI to the reaction, providing evidence for a putative mechanism involved in blocking JHMV infection in OL (Wilson *et al.*, 1990). As might be expected from these findings, the block to the process of MCV uncoating, relating to the dephosphorylation of N in mature OL, can be circumvented by transfection with genomic JHMV RNA (K. Kalicharran and S. Dales, unpublished data).

C. Immunity as a Factor Regulating Pathogenesis and Disease

Both cellular and humoral components of immunity modulate the disease process due to CNS infections by MCV. Protection against acute, lethal enceph-

FIGURE 1. Modulation of R I and R II in primary rat astrocytes and oligodendrocytes during treatment with 1 mM dbcAMP. The concentrations of the regulatory subunits in cytosol ($100,000 \times g$) fractions from astrocytes and oligodendrocytes were determined by binding of 8-azido- $[^{32}P]$ -cAMP. A densitometer tracing (A) made from an autoradiogram (B), obtained after 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, enabled a comparison of the time-related changes in the R I regulatory subunit. Absorbance units have been normalized to the band of greatest density (oligodendrocytes, 5 days posttreatment). (From Beushausen *et al.*, 1987.)

EARLY CELL-VIRUS INTERACTIONS

FIGURE 2. Diagrammatic representation of the early events connected with MCV uncoating. Dephosphorylation of NC protein is proposed as a necessary event for initiation of processing of NC to facilitate conformational relaxation of the genome and/or release of NC from the genome to allow the expression of subsequent virus-specified functions.

alitis is achieved by passive immunization with MAb against viral envelope glycoproteins S, M, and HE. Such immunization, however, fails to arrest the delayed, demyelinating form of disease and may in fact promote it (Fleming *et al.*, 1989). Our own studies with rat tetracephalic cultures suggest that the subacute disease with demyelination may occur only after survival of the acute, encephalitic phase of disease (Pasick and Dales, 1991). An antibody-mediated shift in tropism is indicated by the interference with neuronal, but not OL infections, when MAb against S or HE is employed (Buchmeier *et al.*, 1984; Yokomori *et al.*, 1992). This implies that complement-mediated cytolysis or antibody-dependent, cell-mediated cytotoxicity (ADCT) may eliminate infected targets following the mobilization and infiltration of mononuclear cells. In addition, an increase of B and plasma cells as well as IgG⁺ complement deposits within demyelinating lesions of WL rats (Zimprich *et al.*, 1991) is consistent with the possible role of ADCT.

During acute phases of murine CNS infection by JHMV, the T-cell response may be crucial in mediating clearance of virus and arrest of the disease process. Upon adoptive transfer of CD4⁺, Lyt2-cloned T cells specific for JHMV, C57BL/6 mice are protected from developing lethal encephalitis by an major histocompatibility complex II (MHC-II)-restricted delayed type hypersensitivity (DTH) response (Stohlman *et al.*, 1986, 1988), without a reduction in virus titer in the CNS. Such protected mice subsequently manifest OL demyelinating disease. The CNS concentrations of anti-JHMV antibodies are not altered in the above T-cell recipients or in WF rats with demyelinating lesions (Sorensen *et al.*, 1984b). It can be concluded from these data that the local synthesis of antiviral antibodies within the CNS does not prevent demyelination. The adoptive transfer of T-cell clones specific for JHMV can also protect

against encephalitis by reducing virus replication in neurons (Stohlman *et al.*, 1986). Furthermore, some data suggest that the clearance of JHMV from the CNS of mice appears to involve both CD4⁺ and CD8⁺ T cells (Sussmann *et al.*, 1989; Williamson and Stohlman, 1990; Yamaguchi *et al.*, 1991). These results are in line with the evocation of a CTL response against the carboxy-terminal end of N (Stohlman *et al.*, 1992). Another study, however, failed to demonstrate cytotoxic lymphocytes (CTLs) against viral components in C57BL/6 mice, although other T-helper cell responses were detected (Mobley *et al.*, 1992). Complementary studies employing athymic, nude mice and rats or animals treated with immunosuppressing agents confirmed the involvement of cellular immunity in the disease process. They showed that, relative to control animals, gray matter and neuronal involvement is enhanced during T-cell deficiency, resulting in acute encephalitic rather than demyelinating, white matter disease (Sorensen *et al.*, 1982, 1987b; Zimmer and Dales, 1989; Pasick *et al.*, 1992).

Postweanling rats challenged with JHMV do not exhibit any disease symptoms, but on histological examination show signs of limited CNS histopathology and some virus expression. The age-limited resistance does not apply to athymic nude (nu/nu) rats or animals treated with the immunosuppressants cyclophosphamide and cyclosporin A (Sorensen *et al.*, 1987b; Zimmer and Dales, 1989). Rats challenged with virus even beyond 70 days of age develop a late-onset encephalitic form of disease that is rapidly fatal. Histopathology reveals wide dissemination of infection in neuron-rich gray matter but a limited involvement of white matter, where small foci of demyelination develop during the late-onset disease. In adult mice of strains that are normally resistant to A59, MHV3, and JHMV MCV infections, when initiated by the IN and IP routes, immunosuppression promotes a similar, late-onset encephalitis arising from extensive neuronal damage (Sorensen *et al.*, 1982, 1984a,b). It appears that CNS neurons in older rats and mice are fully susceptible to infection but replication and dissemination of MCV progeny is suppressed when cellular immune responses are activated. Presumably, the paucity of susceptible OL targets for MCV infection within the CNS of mature animals results in a limitation of pathogenesis, manifested as very small WM demyelinating lesions (Sorensen *et al.*, 1987b; Zimmer and Dales, 1989).

Consistent with the *in vivo* situation, the susceptibility of neurons can also be demonstrated with cortical explants from rat embryos that are maintained in long-term culture. Unlike explanted OL, which are intrinsically programmed to differentiate within 3 weeks after birth or at an accelerated rate by treatment with dbcAMP (Raff, 1989; Beushausen and Dales, 1985), isolated neurons remain susceptible to persistent JHMV infection for long periods of time and can produce large quantities of virus after pretreatment with dbcAMP (Pasick and Dales, 1991). The late-onset, fulminant infection of neurons in immunosuppressed rats might commence from a reservoir of virus at a foci of latency, perhaps in neurons of the cerebellum and hippocampus (Sorensen and Dales, 1985).

Unlike cellular immunity, the humoral immune responses that occur within the CNS compartment following infection of WF and WL rats fail to suppress virus replication or interrupt the development of disease (Sorensen *et al.*, 1984b). In Brown Norway rats, a vigorous humoral immunity within the

CNS develops after JHMV infection, which could explain why there is a suppression of disease and virus replication in this model (Dörries *et al.*, 1987).

The infection of macrophages and lymphotropism of MHV3 during infection of partially susceptible (C57BL/6 $\times$ A/J) F_1 mice initially causes a low-grade infection of the liver, then of the brain. Upon spreading to the CNS, the infection produces paralytic symptoms, sometimes lasting for periods as long as 1 yr. Lymphotropism is associated with a progressive decline of immunoglobulin production during the initial 3 months, then a return to normal levels (Leray *et al.*, 1982). After introduction of JHMV by IN inoculation, adult BALB/c mice become protected against challenge for 12 months. This is due to antibodies raised against the initial inoculation (Barthold and Smith, 1989). The virus may incite an autoimmune disease process in WL rats and SJL/J mice, which follows myelin destruction, as described in Section IV. Autoimmunity was postulated to involve expression of the MHC-I associated (Ia) determinant on AS when these cells become differentiated into antigen-presenting cells due to the presence of JHMV (Massa and ter Meulen, 1987; Calder *et al.*, 1988). This phenomenon does not appear to pertain in mice, in which only class I H-2 antigen presentation is observed in AS and OL (Suzumura *et al.*, 1986, 1988). The presentation of class I H-2 required continuous presence or formation of viable A-59 virus and is mediated by factors produced in glial cells, probably AS (Lavi *et al.*, 1989).

IV. GENETIC VARIABILITY OF VIRUS AND HOST AS DETERMINANTS OF PATHOGENESIS AND DISEASE

Differences in the cytopathology and disease process in animals are greatly affected by the genotypes of both the virus and the host. During interactions with cell targets, the S peplomer is most probably the major determinant of tropism, virulence or attenuation, and cytopathology. The host controls the disease process and susceptibility or resistance to infection at the cellular and organismic level, including responses by the immune system.

A. Relationship of the CV Replication Strategy to Genetic Variability

An understanding of the CV replication strategy can explain why these agents have the capacity to evolve rapidly into diverse genetic variants. Thus, transcription by means of discontinuous, leader-primed synthesis (Lai, 1986), as well as the occurrence of subgenomic replicative intermediates (Sethna *et al.*, 1989; Sawicki and Sawicki, 1990), provide possible mechanisms for highly efficient recombination among the nonsegmented CV genomes (Lai *et al.*, 1985). Based on the currently available CV genomic and mRNA sequences, it is possible to explain the genesis of diversity observed among MCV progeny following passage through cells in culture or during CNS infection of animals. Also, in the case of HCV, circulating among human populations, genetic variability could arise by repeated OC43 and 229E reinfections of the upper respiratory tract (MacNaughton, 1982; Reed, 1984).

The occasional association of OC43 infections of humans with neurological symptoms (Hellevi and Hovi, 1980) and the induction of a demyelinating disease process by MCV in rodents and monkeys have a bearing on the search for an etiologic infectious agent in multiple sclerosis (MS). However, the claim that two CV isolates, designated SK and SD, had originated from the tissue of MS patients (Mendelman *et al.*, 1983) remains questionable. Sensitive antigen detection (Hasony and MacNaughton, 1982) and nucleic acid probes (Weiss, 1983; Weiss and Leibowitz, 1983) that are able to discriminate between HCV and MCV have grouped SK and SD viruses with MCV (Weiss, 1983).

B. Role of S in Pathogenesis and Disease

The S peplomer, which is involved in attachment, penetration, syncytio-genesis, and other aspects of cytopathology, appears to be one of the most variable component of coronavirions. In addition to molecular diversity in S from the various CV strains infecting mammals, variability in the MCV S protein arises during passage through rodents, generating antigenic and pathogenicity variants, as summarized in Table II. An important location for molecular modulation could be a hypervariable RNA sequence identified in the S gene (Parker *et al.*, 1989). During replication, this region may either be a preferred crossing over site or a site of strong selection, thereby giving rise to recombinants of S in which modifications, including deletions, are introduced frequently.

The unquestionable role of S in cytopathogenicity and neuroattenuation was demonstrated when S was expressed by means of a vaccinia virus recombinant vector. This experiment of Daya *et al.* (1989) demonstrated unequivocally that S, by itself, can induce the same cytopathology as the MCV from which it was derived. Also, it has been possible to identify separate regions on S associated with pathogenicity and other functions (Table II). These various biological activities of S were mapped using panels of MAbs able to affect individual functions of this G-P. Thus, three topologically distinct antigenic sites, eliciting protective cellular or humoral responses in mice, were identified on the S protein of JHMV by Talbot *et al.* (1984). The survival of mice was associated with decreased virus production and confinement of the infection to neurons, sparing the glia (Buchmeier *et al.*, 1984).

Another comparative study on the MCV strains MHV-1, -3, -5, JHM, and A59 demonstrated the presence of five antigenic sites, A through E, among which B is involved with neurotropism and C with virulence (Talbot and Buchmeier, 1985). Such analyses also uncovered polymorphism in the S protein of JHMV variants isolated after escaping neutralization by MAb (Dalziel *et al.*, 1986; Buchmeier *et al.*, 1988). Some isolates obtained in this manner were attenuated and others more virulent when compared with the parental JHMV. Attenuation was manifested through a requirement for larger inocula to initiate CNS disease and a change in preferential tropism from neurons to OL. Thus, a paralytic, demyelinating disease rather than an acute encephalitis was elicited (Buchmeier *et al.*, 1988). Controlled dual infection with JHMV and A59 has also

generated recombinants in the S gene. Epitope mapping carried out with MAb demonstrated that the critical determinant(s) for neuropathogenicity occur at the C terminal one third of the S protein (Makino *et al.*, 1987; Gallagher *et al.*, 1990). In some variants, attenuation is linked with a *ts* phenotype of the virus, as in the case of one A59 isolate that replicated at a normal rate in Sac(-) cells but behaved as *ts* in glial cultures and was mildly neuropathogenic in mice (van Berlo *et al.*, 1986; Koolen *et al.*, 1983). From this and other findings, it becomes evident that a temperature restriction regulating CV infection can be exerted by the neural cell host and is not due to thermosensitivity of the virus, as discussed in Section II.G (Lucas *et al.*, 1978; van Berlo *et al.*, 1986; Koolen *et al.*, 1983).

It is possible that some phenotypic variations of S are due to point mutations alone, but it is known that during passage through neural cells in explants or within the CNS, the S protein of the emerging stable variants undergoes considerable changes, as described in Table II. One JHMV isolate, passaged in C1300 neuroblastoma, possessed a deletion in S and coincidentally became nonsyncytiogenic (Lavi *et al.*, 1990). In another JHMV isolate from the CNS that could replicate more efficiently in AS, the mRNA encoding S was larger (Taguchi *et al.*, 1986). In another study of six JHMV isolates from mouse CNS, four were shown to possess larger and two smaller than normal length S genes. In the smaller S variants, sequence deletions were linked with an attenuated phenotype producing a subacute demyelinating disease in mice (Taguchi and Fleming, 1989).

Similar modulations in S of JHMV were generated by passage through the CNS of rats. One report described a stable change to a larger S polypeptide (Taguchi *et al.*, 1985). As described in Table II, genetically stable JHMV variants have been isolated and different phenotypes for the induction of neurological disease in the white and gray matter of the CNS can be recognized (Baybutt *et al.*, 1984; Morris *et al.*, 1989). One such attenuated isolate from spinal cord (SC), termed AT11f, has a deletion in S, is highly fusogenic for cells in culture, and rather specific for inducing SC demyelination (Morris *et al.*, 1989). AT11f overcomes the resistance of explanted glial cells in adult SJL/J mice, apparently, unlike infection with parental JHMV, because dissemination of the AT11f progeny is not as restricted and high titers are produced (Pasick *et al.*, 1992). Thus, an alteration in S has made this isolate more, not less, virulent for mouse neural cells. A double mutant attenuated in both encephalitic and demyelinating potential contained a point mutation within S2 plus a deletion in the hypervariable region of S1 (Fleming *et al.*, 1986, 1987). This finding led to the conclusion that the role of S in demyelination may involve multiple determinants on this molecule (Wang *et al.*, 1982). Evidently, care must be taken in defining attenuation and virulence as it pertains to diseases produced by these variants.

C. Influence of the Genetic Constitution of the Host

There is abundant documentation that the resistance or susceptibility to MCV-induced disease process is controlled by the genetic determinants of the host. With mice, the determinants have been recognized at the cellular level.

TABLE II. Relationship between Changes in S of JHMV and CNS Disease^a

Parental virus strain	Variant	Basis of selection	Alteration in the molecule	Neuropathology observed	References
JHMV	AT11f cord	Spinal cord of WF rate with demyelinating encephalomyelitis	441-nucleotide deletion in hypervariable region of S1 segment; 738-nucleotide deletion in the HE gene	Neuroattenuated; induces acute demyelinating disease	Morris <i>et al.</i> (1989); La Monica <i>et al.</i> (1991)
	2.2-V-1	Neutralizing resistance to MAb	Single point mutation at nucleotide 3340 in S2 segment	Neuroattenuated; induces subacute demyelinating disease	Fleming <i>et al.</i> (1986); Wang <i>et al.</i> (1992)
	7.2-V-1	Neutralization resistance to MAb	Two point mutations at nucleotides 1766 and 1950 in S1	Retains encephalitogenic potential	Fleming <i>et al.</i> (1986); Wang <i>et al.</i> (1992)
	2.2/7.2-V-2	Double neutralization resistance	Deletion spanning nucleotides 1523 to 1624 in S1 hypervariable region plus point mutation at nucleotide 3340 in S2	Neuroattenuated for both encephalitic and demyelinating potential	Fleming <i>et al.</i> (1986); Wang <i>et al.</i> (1992)
	c1-2	Brain of Lewis with acute encephalitis	15-kDa enlargements of S protein	Enhanced neurovirulence with widespread neuronal and glial involvement in weanling Lewis rats	Matsubara <i>et al.</i> (1991); Taguchi <i>et al.</i> (1985)
CNSV	Isolated from cultured rat neural cells		Similar though not identical enlargement of S found in c1-2	Enhanced tropism for astrocytes in vitro, enhanced neurovirulence in vivo	Matsubara <i>et al.</i> (1991); Taguchi <i>et al.</i> (1986)

N3	Chemical mutagenesis <i>ts</i> mutant	RNA negative	Neuronal infection without encephalitis; induces meningitis and demyelination	Robb <i>et al.</i> (1979)
ts43	Chemical mutagenesis <i>ts</i> mutant	—	Persistent infections of rat glial cultures with predominant involvement of astrocytes; demyelinating disease with inflammatory infiltrates	Massa <i>et al.</i> (1988)
MHV-4	V5A 13.1 Neutralization resistance to S MAbs	426-nucleotide deletion in S1 involving nucleotides 1298 to 1723	Neuroattenuated; increased LD ₅₀ ; demyelination associated with moderate inflammatory cell infiltration; apparent loss of neuronal tropism	Dalziel <i>et al.</i> (1986); Gallagher <i>et al.</i> (1990); Parker <i>et al.</i> (1989)
MHV-4	V4B11.3 Neutralization resistance to S MAbs Chemical mutagenesis <i>ts</i> mutant	477-nucleotide deletion in S1 involving nucleotides 1285 to 1761	Neuroattenuated; acute demyelinating disease without associated inflammatory cell infiltration	Haspel <i>et al.</i> (1978); Knobler <i>et al.</i> (1982)

^aAdapted from Pasick and Dales (in press).

Notably, SJL/J mice resist infection by JHMV but not by the related MHV3. This distinction between virus strains becomes clear when different primary cell types are challenged, including neurons, macrophages (Knobler *et al.*, 1981), AS, and OL (Wilson and Dales, 1988). In contrast to SJL/J mice, A/J adults and their peritoneal exudate macrophages are resistant to MHV3. By challenging IN the hybrid mice crosses SJL/J $\times$ CD.1 and A/J $\times$ DBA and explanted cells from these crosses, it can be demonstrated (as shown in Table III) that resistance is manifested as a recessive trait (reviewed in Wilson and Dales, 1988). This result is consistent with the idea that a specific, cellular protease is required to activate S by cleavage to S₁ and S₂, and thus promote the spread of infection. However, the disease pattern produced with the virus when inoculated into F₁ mice is unlike that expected from fully susceptible mouse strains. For example, IP challenge of the F₁ A/J $\times$ DBA cross with MHV3, rather than inducing a fulminant visceral and liver disease, instead elicits late-onset neurological symptoms with paralysis (Sorensen *et al.*, 1982; Le Prévost *et al.*, 1975). Evidently some additional factor(s) is controlling virus expression and influencing the *in vivo* disease process.

It has been suggested that any variable that influences survival of the animal host, following virally induced encephalitis, results in the subsequent manifestation of a demyelinating form of disease (Fleming *et al.*, 1989). Our own studies indicate that subacute demyelination may occur after the host has undergone a transitory and limiting form of encephalitis (Pasick and Dales, 1991).

Genetic variability of the cellular immune response could be one of such factor determining the outcome of the disease process. Peritoneal macrophages

TABLE III. Comparison of Replication in Glial Cultures from Purebred and Hybrid Mice^{a,b}

Mouse strain from which culture originated	Time after inoculation (hr)	Titer (10 ² PFU/ml) ^c		
		JHMV ^c	MHV3 ^d	A59 ^d
SJL	12	0.17 $\pm$ 0.29 (3)	590 $\pm$ 114 (3)	6 $\pm$ 2 (6)
	24	23 $\pm$ 12 (14)	435 $\pm$ 292 (14)	13 $\pm$ 0.5 (6)
	48	48 $\pm$ 20 (14)	886 $\pm$ 62 (10)	2 $\pm$ 0.6 (3)
	72	22 $\pm$ 9 (12)	475 $\pm$ 119 (6)	ND ^e
CD.1	12	350 $\pm$ 216 (5)	77 $\pm$ 12 (4)	ND
	24	3,260 $\pm$ 2,110 (13)	778 $\pm$ 30 (11)	21,300 $\pm$ 19,600 (3)
	48	9,400 $\pm$ 500 (9)	3,340 $\pm$ 2,300 (7)	95,800 $\pm$ 2,700 (3)
(CD.1 $\times$ SJL)F ₁	24	961 $\pm$ 34 (5)	1,120 $\pm$ 730 (5)	ND
	48	6,260 $\pm$ 4,320 (5)	2,400 $\pm$ 2,000 (5)	ND
	72	943 $\pm$ 480 (5) ^f	213 $\pm$ 52 (5) ^f	ND

^aFrom Wilson and Dales (1988).

^bPrimarily oligodendrocytes and astrocytes (35).

^cThe values are means with standard deviations. The number of cultures tested is shown in parentheses.

^dMOI, 1 PFU per cell.

^eND, not determined.

^fDecrease in titers was attributed to rapid cell killing.

from a substrain of C₃H mice, normally resistant to MCV, became susceptible if the host had been pretreated with hydrocortisone, implying that a lymphokine-induced resistance was involved (Taylor *et al.*, 1981). Infection of susceptible C57BL/6 mice with MHV3 may abrogate or depress cellular immunity directly because the virus is lymphotropic, hence immunosuppressive, and causes the spleen and thymus to atrophy (Lamontagne *et al.*, 1989). The role of macrophages in the MHV3 disease process can be demonstrated in the adoptive transfer of resistance by means of bone marrow cells from resistant into susceptible X-irradiated mouse recipients (Lamontagne *et al.*, 1989; Dupuy *et al.*, 1984).

Among rats, strain WF has a more prolonged age-related susceptibility to inoculation with JHMV than WL and other strains (Sorensen *et al.*, 1982). The tropism of this virus for immature OL *in vitro* (Beushausen and Dales, 1985; Wilson *et al.*, 1986; Beushausen *et al.*, 1987) and the documented, extended duration of susceptibility to JHMV in postnatal, preweanling WF rats may be connected. Since the maturation of the CNS in this strain is slower, presumably due to genetic deficiency of circulating growth hormone (reviewed in Sorensen *et al.*, 1980), a greater number of infectable OL progenitors might be present for a longer duration in WF versus WL rats. As is the case with the mouse, rats also manifest genetic variation of immune responses, which are superimposable on virus-target cell interactions. An autoimmune type of disease, similar to experimental allergic encephalomyelitis (EAE), was recognized following JHMV infection of WL rats (Watanabe *et al.*, 1983; Wege *et al.*, 1984). This strain is readily inducible for EAE by inoculation with myelin components. However, despite similarities in appearances of demyelinating lesions evident in WL and WF rats, EAE cannot be induced with myelin antigens in WF rats (Zimmer and Dales, 1989). These combined data indicate that JHMV infection of WL rats may elicit a primary demyelination due to OL infection, as well as an EAE-like disease, provoked by circulating myelin antigens released by cytolysis of infected OL. This duality of disease processes may occur in WL rats, perhaps because they are endowed with a high frequency of T-cell responders to myelin basic protein.

In rats, naturally or deliberately made deficient in cellular immunity, susceptibility to JHMV-related CNS disease becomes extended indefinitely beyond weaning. Athymic nu/nu rats undergo a late-onset acute encephalitis with only a limited white matter involvement, evident by the presence of small lesions (Sorensen *et al.*, 1987b). The disease process is primarily connected with fulminant gray matter neuronal infections, which cannot be kept in check due to a genetic deficiency of cellular immunity. Humoral immune responses, although they do occur locally within the CNS of JHMV-infected WL and WF rats, appear not to influence the demyelinative disease process (Sorensen *et al.*, 1984b; Dörries *et al.*, 1986). On the other hand, a vigorous humoral immunity detected in the CNS of BN rats could explain why the disease is suppressed in this strain (Dörries *et al.*, 1987).

From the above considerations, it is evident that genetic variability of the virus and the host can result in differing diseases in animals and varying cytopathic responses when infections are carried out *in vitro*. Within the animal there are additional important factors, most notably the immune system,

that also determine the nature of pathogenesis and type of disease that develops.

V. SUMMARY

Based on the information reviewed in this chapter, it becomes clear that both the virus and host are involved in determining the outcome of CNS infections by MCV. The current information is consistent with the idea that virus tropism for specific cell types within the CNS is profoundly influenced by: (1) the state of maturation of the CNS, i.e., by cell lineages and parameters controlling differentiation; (2) immune responses; and (3) possibly, evolution of genetic variants of the virus. Thus, pathogenesis and neurological disease in this complex rodent model of virus infection become a manifestation of an interplay between the above three, equally important, parameters.

VI. REFERENCES

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